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Original Paper

Clinical and Biological Implications of Mutational Spectrum in Acute Myeloid Leukemia of FAB Subtypes M0 and M1

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Key Words
Acute myeloid leukemia • M0 and M1 • Next generation sequencing • Mutational spectrum • Prognosis

Abstract
Background/Aims: Acute myeloid leukemia (AML) of French-American-British (FAB) subtypes M0 and M1 are both poorly differentiated AML, but their mutational spectrum and molecular characteristics remain unknown. This study aimed to explore the mutational spectrum and prognostic factors of AML-M0 and M1. ***Methods:*** Sixty-five AML patients derived from The Cancer Genome Atlas (TCGA) database were enrolled in this study. Whole-genome sequencing was performed to depict the mutational spectrum of each patient. Clinical characteristics at diagnosis, including peripheral blood (PB) white blood cell counts (WBC), blast percentages in PB and bone marrow (BM), FAB subtypes and the frequencies of known recurrent genetic mutations were described. Survival was estimated using the Kaplan-Meier methods and log-rank test. Univariate and multivariate Cox proportional hazard models were constructed

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for event-free survival (EFS) and overall survival (OS), using a limited backward elimination procedure. ***Results:*** Forty-six patients had more than five recurrent genetic mutations. *FLT3* had the highest mutation frequency (n=20, 31%), followed by *NPM1* (n=18, 28%), *DNMT3A* (n=16, 25%), *IDH1* (n=14, 22%), *IDH2* (n=12, 18%), *RUNX1* (n=11, 17%) and *TET2* (n=7, 11%). Univariate analysis showed that age ≥60 years and *TP53* mutations had adverse effect on EFS (*P*=0.015, *P*=0.036, respectively) and OS (*P*=0.003, *P*=0.004, respectively), WBC count ≥50×10^9/L and *FLT3-ITD* negatively affected EFS (*P*=0.003, *P*=0.034, respectively), whereas *NPM1* mutations had favorable effect on OS (*P*=0.035) and allogeneic hematopoietic stem cell transplantation (allo-HSCT) on EFS and OS (all *P*<0.001). Multivariate analysis suggested that allo-HSCT and *NPM1* mutations were independent favorable prognostic factors for EFS and OS (all *P*<0.05), WBC count ≥50×10^9/L was an independent risk factor for EFS (*P*=0.002) and *TP53* mutations for OS (*P*=0.043). ***Conclusions:*** Our study provided new insights into the mutational spectrum and molecular signatures of AML-M0 and M1. We proposed that *FLT3-ITD*, *NPM1* and *TP53* be identified as markers for risk stratification of AML-M0 and M1. Patients with AML-M0 and M1 would likely benefit from allo-HSCT.

Introduction

Acute myeloid leukemia (AML) is a heterogeneous malignancy characterized by clonal expansion and differentiation arrest of myeloid progenitors in the bone marrow and peripheral blood; historically AML had poor prognosis [1]. Optimizing treatment based on accurate diagnosis and prognostic evaluation in individual patients is particularly important due to disease heterogeneity [2]. Recently, next generation sequencing (NGS) has shown great potential in AML diagnosis and risk stratification because of its massive parallel sequencing ability and high throughput multiplexing capacity [3]. NGS helped characterizing several recurrent somatic mutations in AML, drawing the details of its mutational spectrum [4]. The growing list of mutations involve prognosticators such as *NPM1*, *FLT3-ITD*, *CEBPA*, *DNMT3A*, *IDH1* and *IDH2*, as well as genes implicated in leukemogenesis, such as *EZH2*, *U2AF1*, *SMC1A* and *SMC3* [5]. A recent study analyzed 1, 540 AML patients by cytogenetic profiling and targeted resequencing of 111 myeloid cancer genes, the patterns of co-occurrence and mutual exclusivities of genetic changes segregated AML patients into 11 nonoverlapping classes, each with a distinct clinical phenotype and outcome [6]. Another study analyzed the genomes of 200 adult AML patients by NGS, and mutations were divided into nine categories. Almost all AML patients had one or more mutations that fell into the nine categories, and a complex interplay of genetic alterations was found [5].

Several decades ago, in order to provide objectivity in the diagnosis of AML that would facilitate comparisons between series of cases, the French-American-British (FAB) Cooperative Group developed a classification system based on conventional morphologic and cytochemical characteristics and divided AML into FAB subtypes (M0-M7) [7], with AML-M0 and M1 being the poorly differentiated subtypes. Although advances in identification of prognostic genetic alterations have facilitated detailed risk stratification [8], currently no research has addressed the mutational spectrum of AML-M0 and M1. It's unclear whether they differ in mutational spectrum and how genetic signatures influence their prognosis. We intended to describe the clinical and molecular prognostic factors for the development of optimal and individualized therapy for AML-M0 and M1 patients.

Materials and Methods

Patients

Sixty-five AML patients derived from The Cancer Genome Atlas (TCGA) database (https://cancergenome.nih.gov/) were enrolled in this study [5], including 19 AML-M0 and 46 AML-M1 patients. Poor-risk patients each underwent allogeneic hematopoietic stem cell transplantation (allo-HSCT) if

there was no contraindication and a matched donor was available. Many intermediate risk patients also underwent allo-HSCT. The number of patients receiving allo-HSCT was 37 and the rest 28 had chemotherapy only. Whole-genome sequencing was performed to depict the mutational spectrum of each patient. Clinical characteristics at diagnosis, including peripheral blood (PB) white blood cell counts (WBC), blast percentages in PB and bone marrow (BM), French-American-British (FAB) subtypes and the frequencies of known recurrent genetic mutations were described. Detailed descriptions of clinical and molecular characteristics were publicly accessible from the TCGA website. Event-free survival (EFS) and overall survival (OS) were the primary endpoints of this study. EFS was defined as the time from diagnosis to the first event including relapse, death, absence of complete remission or the last follow up. OS was defined as the time from diagnosis to death from any cause or the last follow-up. All patients provided informed consent, and the study protocol was approved by the Washington University Human Studies Committee.

Statistical Analysis

The clinical and molecular characteristics of patients were summarized using descriptive statistics. Data sets were described with median and/or range. Survival was estimated using the Kaplan-Meier method and the log-rank test. Univariate Cox proportional hazards models were used to identify clinical and molecular variables associated with survival. Multivariate proportional hazards models were constructed for EFS and OS, using a limited backward elimination procedure. $P<0.05$ was considered statistically significant for all analyses. All statistical tests were two-sided and were performed by SPSS software 20.0 and GraphPad Prism software 5.0.

Results

Demographic and biological characteristics of the patients

The demographic and biological characteristics of the patients were summarized in Table 1. Median age was 58 (range 18-88) years, with 31 cases older than 60. Thirty-seven cases were men. Nineteen patients were AML-M0 and 46 were AML-M1. The median WBC count at diagnosis was 19.8×10^9/L, and in 16 cases it was $\geq50\times10^9$/L. Forty-eight patients had BM blast percentage more than 70% and 28 had PB blasts more than 70%. Thirty-four patients had abnormal karyotypes. Sixty patients had intermediate or poor risk AML. Chemotherapy was differed in two patients due to old age and poor functional status. Thirty-seven patients received HSCT, of which 24 cases achieved complete remission. Forty-six patients had more than five recurrent genetic mutations. *FLT3* had the highest mutation frequency (n=20, 31%), followed by *NPM1* (n=18, 28%), *DNMT3A* (n=16, 25%), *IDH1* (n=14, 22%), *IDH2* (n=12, 18%), *RUNX1* (n=11, 17%) and *TET2* (n=7, 11%) (Fig. 1).

Table 1. Clinical and molecular characteristics of the patients Abbreviations: FAB, French American British; WBC, white blood cell; BM, bone marrow; PB, peripheral blood; HSCT, hematopoietic stem cell transplantation; MUD, matched unrelated donor; Allo, allogeneic; Auto, autologous

Characteristics	Median (range) or N/%
Age (years)	58 (18-88)
<60	34/52.3
≥60	31/47.7
Gender	
Male	37/56.9
Female	28/43.1
Race	
Caucasian	44/67.7
Others	21/32.3
FAB subtypes	
M0	19/29.2
M1	46/70.8
WBC count/×10⁹/L	19.8 (0.7-297.4)
<50	49/75.4
≥50	16/24.6
BM blasts/%	81 (32-100)
<70	17/26.2
≥70	48/73.8
PB blasts/%	55 (0-98)
<70	37/56.9
≥70	28/43.1
Karyotype	
Normal	30/46.9
Abnormal	34/53.1
Risk	
Good	4/6.2
Intermediate	40/62.5
Poor	20/31.3
Recurrent gene mutations	6 (0-12)
<5	19/29.2
≥5	46/70.8
MLL-PTD	
Positive	4/6.2
Negative	61/93.8
FLT3	
FLT3-ITD	15/23.1
FLT3-TKD	5/7.7
Wild type	45/69.2
NPM1	
W288	18/27.7
Wild type	47/72.3
DNMT3A	
R882	6/9.2
Non-R882 mutations	10/15.4
Wild type	49/75.4
IDH1	
R132	14/21.5
Wild type	51/78.5
IDH2	
R140	9/13.9
R172	3/4.6
Wild type	53/81.5
RUNX1	
Mutation	11/16.9
Wild type	54/83.1
TET2	
Mutation	7/10.8
Wild type	58/89.2
CEBPA	
Single-mutation	6/9.2
Wild type	59/90.8
TP53	
Mutation	5/7.7
Wild type	60/92.3
PTPN11	
Mutation	5/7.7
Wild type	60/92.3
MT-CO2	
Mutation	5/7.7
Wild type	60/92.3
ASXL1	
Mutation	5/7.7
Wild type	60/92.3
NRAS	
Mutation	4/6.2
Wild type	61/93.8
KRAS	
Mutation	4/6.2
Wild type	61/93.8
TTN	
Mutation	4/6.2
Wild type	61/93.8
STAG2	
Mutation	4/6.2
Wild type	61/93.8
HSCT	
MUD	21/56.8
Sib allo	12/32.4
Auto	4/10.8

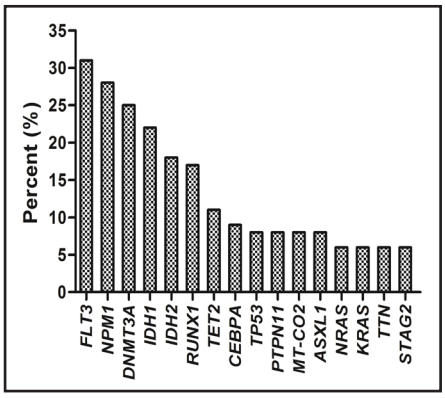

Fig. 1. The mutational spectrum of the patients. FLT3 had the highest mutation frequency (n=20, 31%), followed by NPM1 (n=18, 28%), DNMT3A (n=16, 25%), IDH1 (n=14, 22%), IDH2 (n=12, 18%), RUNX1 (n=11, 17%) and TET2 (n=7, 11%). In addition, CEBPA, TP53, PTPN11, MT-CO2, ASXL1, NRAS, KRAS, TTN and STAG2 also had more than 5% mutation frequency.

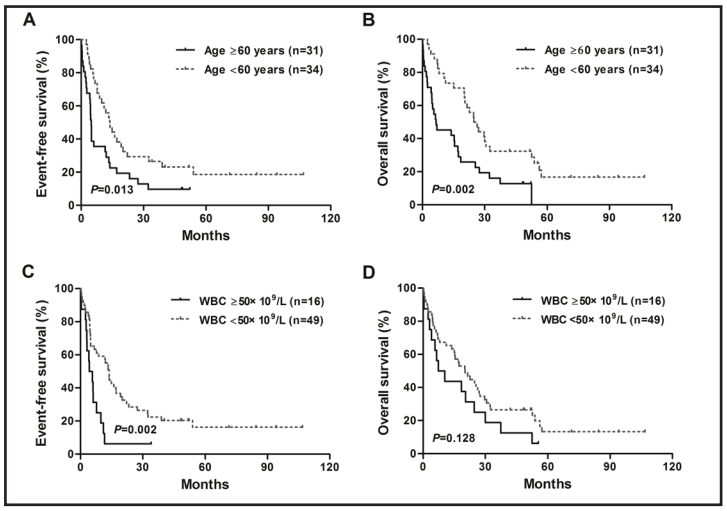

Fig. 2. Kaplan-Meier curves of EFS and OS based on clinical parameters. (A, B) Patients older than 60 years had shorter EFS and OS than those younger than 60 years. (C) Patients with WBC count ≥50×10^9/L had shorter EFS than those with WBC count <50×10^9/L. (D) WBC count had no effect on OS.

Comparison of EFS and OS between different clinical and molecular characteristic groups

EFS and OS of different age (≥60 vs. <60 years), WBC count (≥50 vs. <50×10^9/L), BM blasts (≥70% vs. <70%), PB blasts (≥70% vs. <70%), allo-HSCT (yes vs. no), *FLT3-ITD* (positive vs. negative), and the mutation status of other common AML mutations (*NPM1*, *DNMT3A*, *IDH1*, *IDH2*, *RUNX1*, *CEBPA*, *TP53*, *PTPN11*, *MT-CO2*, *ASXL1*, *NRAS*, *KRAS*, *TTN* and *STAG2*, mutated vs. wild type), were compared with the Kaplan-Meier method and the log-rank test, as listed in Table 2. Older patients (age ≥60) had shorter EFS and OS (P=0.013, P=0.002, respectively, Fig. 2A and 2B). WBC count ≥50×10^9/L negatively affected EFS (P=0.002, Fig. 2C). Positive *FLT3-ITD* was associated with shorter EFS (P=0.031, Fig. 3A). Patients with *TP53* mutations had shorter EFS and OS (P=0.028, P=0.002, respectively,

Table 2. Comparison of EFS and OS between different clinical and molecular characteristic groups. Abbreviation: EFS, event-free survival; OS, overall survival; WBC, white blood cell; BM, bone marrow; PB, peripheral blood; Allo-HSCT, allogeneic hematopoietic stem cell transplantation

Variables	EFS		OS	
	χ^2	P-value	χ^2	P-value
Age (≥60 vs. <60 years)	6.181	0.013	9.601	0.002
WBC (≥50 vs. <50×10^9/L)	9.407	0.002	2.318	0.128
BM blasts (≥70% vs. <70%)	0.030	0.863	0.229	0.632
PB blasts (≥70% vs. <70%)	0.221	0.638	0.358	0.550
FLT3-ITD (positive vs. negative)	4.672	0.031	1.524	0.217
NPM1 (mutated vs. wild type)	1.594	0.207	4.609	0.032
DNMT3A (mutated vs. wild type)	0.003	0.955	0.611	0.434
IDH1 (mutated vs. wild type)	2.953	0.086	3.449	0.063
IDH2 (mutated vs. wild type)	0.632	0.427	0.065	0.798
RUNX1 (mutated vs. wild type)	1.049	0.306	3.779	0.052
TET2 (mutated vs. wild type)	0.070	0.792	0.017	0.897
CEBPA (mutated vs. wild type)	0.444	0.505	0.368	0.544
TP53 (mutated vs. wild type)	4.833	0.028	9.870	0.002
PTPN11 (mutated vs. wild type)	0.001	0.973	0.095	0.757
MT-CO2 (mutated vs. wild type)	0.048	0.827	0.416	0.519
ASXL1 (mutated vs. wild type)	0.002	0.960	0.987	0.321
NRAS (mutated vs. wild type)	0.006	0.939	0.009	0.922
KRAS (mutated vs. wild type)	0.056	0.814	0.179	0.673
TTN (mutated vs. wild type)	0.289	0.591	0.607	0.436
STAG2 (mutated vs. wild type)	0.459	0.498	1.011	0.315
Allo-HSCT (yes vs. no)	14.048	<0.001	14.656	<0.001

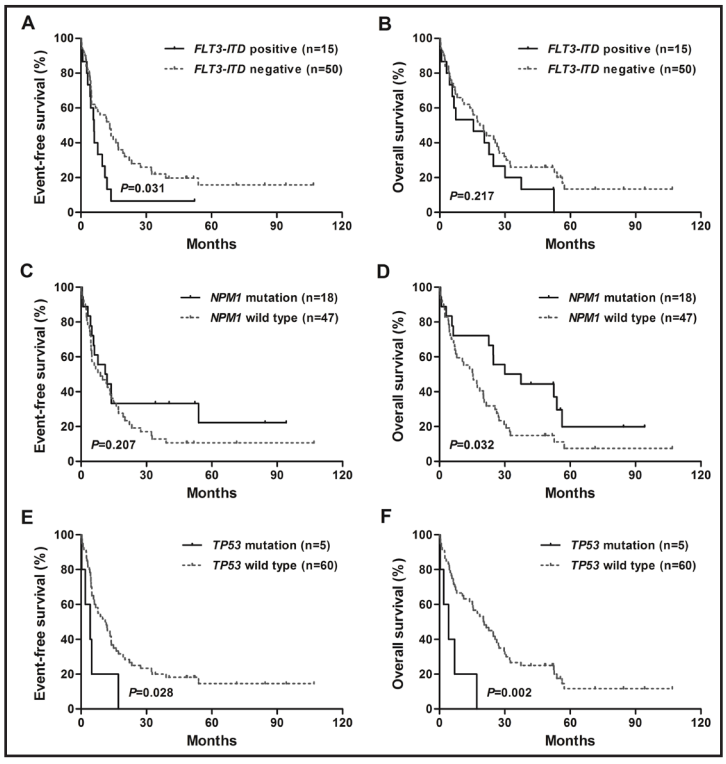

Fig. 3. Kaplan-Meier curves of EFS and OS based on mutated genes. (A) Patients with FLT3-ITD had shorter EFS than negative group. (B) FLT3-ITD had no effect on OS. (C) NPM1 mutations had no effect on EFS. (D) Patients with NPM1 mutations had longer OS than wild type group. (E, F) Patients with TP53 mutations had shorter EFS and OS than wild type group.

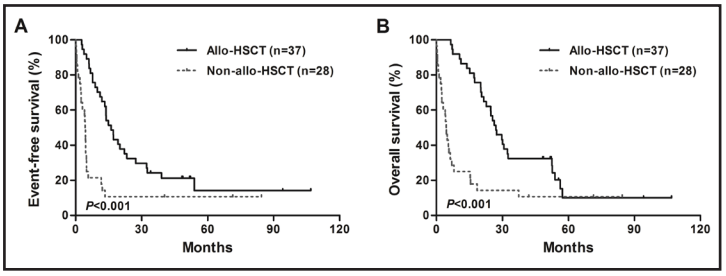

Fig. 4. Kaplan-Meier curves of EFS and OS based on allo-HSCT. (A, B) Patients underwent allo-HSCT had longer EFS and OS than those without allo-HSCT.

Fig. 3E and 3F). Patients with *NPM1* mutations had longer OS (*P*=0.032, Fig. 3D). Furthermore, patients received allo-HSCT had longer EFS and OS (*P*<0.001, *P*<0.001, respectively, Fig. 4A and 4B). Other variables did not demonstrate effect on EFS or OS.

Univariate and multivariate analyses of possible prognostic factors

To further explore the prognostic significance of the aforementioned factors, we did univariate analysis and selected factors that had statistical significance to construct the multivariate COX regression model for EFS and OS. Univariate analysis showed that age ≥60 years was an unfavorable factor for EFS

Table 3. Univariate analysis for EFS and OS. Abbreviation: EFS, event-free survival; OS, overall survival; HR, hazard ratio; CI, confidence interval; WBC, white blood cell; BM, bone marrow; PB, peripheral blood; Allo-HSCT, allogeneic hematopoietic stem cell transplantation

Variables	EFS		OS	
	HR (95%CI)	P-value	HR (95%CI)	P-value
Age (≥60 vs. <60 years)	0.513 (0.300-0.878)	0.015	0.424 (0.243-0.741)	0.003
WBC (≥50 vs. <50×10⁹/L)	0.389 (0.208-0.728)	0.003	0.630 (0.346-1.148)	0.131
BM blasts (≥70% vs. <70%)	0.949 (0.523-1.721)	0.864	1.156 (0.637-2.098)	0.633
PB blasts (≥70% vs. <70%)	0.879 (0.513-1.506)	0.639	1.179 (0.686-2.026)	0.550
FLT3-ITD (positive vs. negative)	1.967 (1.051-3.684)	0.034	1.471 (0.794-2.725)	0.220
NPM1 (mutated vs. wild type)	0.671 (0.358-1.255)	0.211	0.506 (0.269-0.953)	0.035
DNMT3A (mutated vs. wild type)	1.018 (0.553-1.872)	0.955	1.279 (0.689-2.377)	0.436
IDH1 (mutated vs. wild type)	0.552 (0.276-1.100)	0.091	0.525 (0.263-1.048)	0.068
IDH2 (mutated vs. wild type)	0.758 (0.381-1.509)	0.430	1.095 (0.545-2.198)	0.799
RUNX1 (mutated vs. wild type)	1.412 (0.726-2.746)	0.310	1.935 (0.982-3.812)	0.056
TET2 (mutated vs. wild type)	1.121 (0.479-2.624)	0.792	0.946 (0.404-2.211)	0.897
CEBPA (mutated vs. wild type)	1.365 (0.542-3.440)	0.509	1.330 (0.527-3.356)	0.546
TP53 (mutated vs. wild type)	2.727 (1.070-6.951)	0.036	4.166 (1.583-10.958)	0.004
PTPN11 (mutated vs. wild type)	1.016 (0.404-2.556)	0.973	1.156 (0.459-2.913)	0.758
MT-CO2 (mutated vs. wild type)	1.120 (0.404-3.110)	0.827	1.398 (0.503-3.888)	0.521
ASXL1 (mutated vs. wild type)	1.024 (0.406-2.584)	0.960	1.597 (0.629-4.057)	0.325
NRAS (mutated vs. wild type)	1.047 (0.324-3.378)	0.939	0.943 (0.292-3.049)	0.922
KRAS (mutated vs. wild type)	1.130 (0.407-3.135)	0.814	1.247 (0.447-3.484)	0.673
TTN (mutated vs. wild type)	1.322 (0.475-3.680)	0.594	1.503 (0.535-4.222)	0.439
STAG2 (mutated vs. wild type)	0.671 (0.209-2.155)	0.503	0.554 (0.173-1.781)	0.322
Allo-HSCT (yes vs. no)	0.365 (0.211-0.631)	<0.001	0.358 (0.208-0.618)	<0.001

and OS (*P*=0.015, *P*=0.003, respectively), as well as *TP53* mutations (*P*=0.036, *P*=0.004 for EFS and OS, respectively), WBC count ≥50×10^9/L and *FLT3-ITD* negatively affected EFS (*P*=0.003, *P*=0.034, respectively), whereas *NPM1* mutations favorably affected OS (*P*=0.035), and allo-HSCT was a favorable factor for EFS and OS (all *P*<0.001) (Table 3). Multivariate analysis suggested that allo-HSCT was an independent favorable factor for EFS (HR: 0.358, 95% CI: 0.201-0.640, *P*=0.001), the effect was more prominent after adjusting for *NPM1* mutation status (*P*=0.025) and WBC count (*P*=0.002). It was also an independent favorable factor for OS (HR: 0.374, 95% CI: 0.209-0.669, *P*=0.001), with more profound effect after adjusting for *NPM1* (*P*=0.002) and *TP53* mutation status (*P*=0.043) (Table 4).

Table 4. Multivariate analysis for EFS and OS. Abbreviation: EFS, event-free survival; OS, overall survival; HR, hazard ratio; CI, confidence interval; WBC, white blood cell; Allo-HSCT, allogeneic hematopoietic stem cell transplantation

Variables	EFS		OS	
	HR (95%CI)	P-value	HR (95%CI)	P-value
Age (≥60 vs. <60 years)	0.704 (0.386-1.286)	0.254	0.692 (0.371-1.290)	0.246
WBC (≥50 vs. <50×10^9/L)	0.321 (0.156-0.664)	0.002	0.522 (0.240-1.134)	0.101
FLT3-ITD (positive vs. negative)	2.000 (0.949-4.217)	0.069	1.719 (0.742-3.986)	0.207
NPM1 (mutated vs. wild type)	0.439 (0.214-0.900)	0.025	0.291 (0.133-0.637)	0.002
TP53 (mutated vs. wild type)	2.654 (0.956-7.372)	0.061	2.974 (1.034-8.559)	0.043
Allo-HSCT (yes vs. no)	0.358 (0.201-0.640)	0.001	0.374 (0.209-0.669)	0.001

Discussion

AML is a genetically heterogeneous disease resulting from complex interactions among different leukemogenic pathways, so integrated mutational analysis is highly valuable for evaluation [5, 9]. Formerly, the mutational spectrum of AML-M0 and M1 was unclear. In this study, we found that *FLT3-ITD*, *NPM1*, *DNMT3A*, *IDH1*, *IDH2*, *RUNX1* and *TET2* were mutated in more than 10% of all patients with *FLT3-ITD* exhibiting the highest frequency; *CEBPA*, *TP53*, *PTPN11*, *MT-CO2*, *ASXL1*, *NRAS*, *KRAS*, *TTN* and *STAG2* also had more than 5% mutation frequency. This was different from previous reports which showed that *CEBPA*, *NPM1*, *DNMT3A*, *FLT3-ITD*, *NRAS*, *IDH2* and *WT1* were mutated in more than 10% and *CEBPA* mutations were more frequent in intermediate-risk AML [10, 11]. The reported frequency of *CEBPA* mutations in cytogenetically normal AML (CN-AML) was also higher, about 35% [12]. The discrepancy suggested that poorly differentiated AML might have a distinct mutational spectrum.

In uni- and multivariate analyses, we found that age ≥60 years was an adverse factor for EFS and OS, which was consistent with the fact that AML patients younger than 60 years had improved prognosis and approximately 35-40% of them would get cured [13]. WBC count ≥50×10^9/L was also related to shorter EFS and OS, which was consistent with previous finding that WBC count had a significant impact on complete remission rate, EFS and OS in AML patients [14].

FLT3 is a class III family receptor tyrosine kinase that acts as a cytokine receptor for the FLT3 ligand. *FLT3* is strongly expressed in hematopoietic stem cells with important roles in cell survival and proliferation [15]. *FLT3-ITD* was among the most frequent mutations observed in AML, it could activate FLT3 signaling, promoting blast proliferation [16]. Furthermore, *FLT3-ITD* was associated with increased risk of relapse in AML [17]. NPM1 is involved in numerous cellular functions, such as ribosome biogenesis, DNA repair and regulation of apoptosis. *NPM1* mutations were among the most common genetic changes in AML, especially in CN-AML [18]. In the absence of *FLT3-ITD*, *NPM1* mutations were associated with improved outcomes for CN-AML patients. *NPM1* mutations have been associated with chemo-sensitivity to intensive chemotherapy in both young and old patients, which might account for improved outcomes [19]. *NPM1* mutations were also associated with other recurrent genetic abnormalities, such as *DNMT3A*, *FLT3-ITD* and *IDH* mutations [20]. The pattern of co-mutations largely shaped clinical outcomes. *TP53* mutations were rare in patients lacking chromosomal deletions, and it conferred an adverse prognosis with documented chemo-resistance [21, 22]. *TP53* mutations might be responsible for the poor prognosis of complex karyotype AML [23]. Our results showed that *FLT3-ITD* and *TP53* mutations were associated

with shorter EFS and OS, while *NPM1* mutations were associated with favorable prognosis, consistent with previous results. Furthermore, studies indicated that allo-HSCT could lead to better clinical outcomes for patients with unfavorable-risk cytogenetics in the first complete remission [24]. The favorable effect of allo-HSCT was also replicated in our univariate analysis, and the effect was still exist after adjusting for potential confounding factors (age, WBC, *FLT3-ITD*, *NPM1* and *TP53*).

Several limitations need to be acknowledge. First, due to the limited number of our cases, we didn't stratify data more precisely based on factors that could affect the prognosis. So, our results didn't fully account for the effect of mutational spectrum and clinical data on prognosis. Second, our study was a retrospective study which could suffer from inherited biases as opposed to prospective studies.

Conclusion

In summary, we conducted a TCGA database-derived analysis on the mutational profiles and prognosis of AML-M0 and M1 and compared our findings with previous studies. Our study provided new insights into the clinical and biological implications of mutational spectrum in AML-M0 and M1. *FLT3-ITD*, *NPM1* and *TP53* could be incorporated into AML-M0 and M1 risk stratification and these patients would likely benefit from allo-HSCT.

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Disclosure Statement

The authors declare to have no conflict of interests.

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